Endothelin-B receptors mediate intimal hyperplasia in an organ culture of human saphenous vein

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Objective: **Although a number of pharmacologic agents have been shown to reduce intimal hyperplasia in animal models of restenosis, to date no systemic agent has conclusively been shown to be effective in humans. Recently, considerable attention has been directed towards endothelin (ET), a potent vasoconstrictor and a powerful mitogen for vascular smooth muscle cells, as a mediator of intimal hyperplasia. Endothelin-1 has been shown to be mitogenic for human saphenous vein smooth muscle cells, and expression also is elevated in human vein graft stenosis. The aim of this study was the investigation of whether ET receptor antagonists can attenuate neointima formation in a laboratory model of vein graft intimal hyperplasia and the determination of whether the effects are mediated by a specific ET receptor subtype.**

Methods: **We used an organ culture of human saphenous vein, a well-validated model of vein graft intimal hyperplasia. Paired segments of human long saphenous vein were cultured with and without the following antagonists: bosentan, a nonselective ET receptor antagonist; BQ 123, a specific endothelin-A antagonist; or BQ 788, a specific endothelin-B (ET_B) antagonist. After 14 days in the culture, the segments were fixed and processed and the sections were immunostained to facilitate the measurements of neointimal thickness with a computerized image analysis system.**

Results: **The nonselective antagonist bosentan and the ET_B selective antagonist BQ 788 significantly reduced neointima formation by 70% ($P = .001$) and 50% ($P = .03$), respectively, but the ET_A antagonist BQ 123 had no significant effect on the reduction of neointima formation ($P = 1.0$).**

Conclusion: **The results of this study imply an important role for ET as a mediator of human vein graft intimal hyperplasia and imply further that a specific ET_B antagonist may have a therapeutic potential for the prevention of vein graft stenosis. (J Vasc Surg 1998;28:695-701.)**

The development of intimal hyperplasia (IH) poses a significant clinical problem in both the coronary and the peripheral circulations. Thus, up to 40% of the coronary and the peripheral arteries develop restenosis after angioplasty as a result of IH, and up to 35% of saphenous vein bypass grafts develop patency-threatening stenoses within the 1st postoperative year, which again results from localized areas of IH.[1-4] In addition to its vasoconstrictor actions,[5] endothelin-1 (ET-1) is also a powerful mitogen for vascular smooth muscle cells.[6-8] Considerable interest therefore has been directed towards its potential role as a mediator of IH. A role for ET-1 in the development of IH in human vessels is suggested by the finding that ET-1 is mitogenic for human saphenous vein smooth muscle cells[9] and that ET-1 expression is increased in human saphenous vein graft stenoses.[10] In addition, it has recently been shown[11] that ET-1 immunoreactivity is greatly increased in transplant coronary artery disease, a condition characterized by IH. Because most of the ET-1 produced by endothelial cells is released abluminally,[12] increased plasma ET-1 levels would

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suggest a markedly increased production of the peptide at localized sites.

Two main receptor subtypes for ET-1 have been identified and are designated endothelin-A (ET_A) and endothelin-B (ET_B).[13,14] Both receptor subtypes have been shown to coexist in vascular smooth muscle cells from normal human internal mammary arteries and veins[15] and from human coronary arteries and saphenous veins.[16] In the normal human coronary artery and saphenous vein, approximately 10% of the receptors in the media are ET_B and 90% are ET_A, whereas in atherosclerotic vessels, the proportion of ET_B receptors rises to 50%.[17]

In vitro studies in early passaged cultured rat aortic smooth muscle cells have shown that ET-1 induced smooth muscle cell mitogenesis and proliferation are mediated through the ET_A, but not the ET_B, receptor subtype[18] and that the mitogenic activity of ET-1 correlates with ET_A receptor binding and density.[19] However, this mitogenic response seems to be subject to phenotypic modulation because it has also been shown that repeated passaging of smooth muscle cells leads to increased ET_B expression to the extent that selective ET_A antagonism no longer inhibits the mitogenic activity of ET-1.[20] Eguchi et al[21] have shown that the phenotypic change of vascular smooth muscle cells in culture is concommitantly associated with a change in the endothelin (ET) receptor subtype that potentiates mitogenic activity. This suggests that the switching of the ET receptor subtype from A to B during phenotypic change may, in part, contribute to the development of vascular lesions. In support of this hypothesis, Azuma et al[22] showed an increased density of ET_B receptors in the neointima of hyperplastic rabbit arteries. The administration of a specific ET_A antagonist BQ 123 failed to have any effect on the reduction of neointimal thickness, even at concentrations that were sufficient to fully antagonize the ET_A receptors.

In recent years, there has been a growing interest in the role of ET in a wide variety of disease states and a number of ET receptor antagonists have been developed. If ET-1 plays a central role in the cause of IH, then ET receptor antagonists may have therapeutic potential. Indeed, Douglas et al[23] have shown that ET-1 can promote neointima formation, and they have further demonstrated that, in the rat, a potent ET receptor antagonist, SB 209670, reduces IH in injured carotid arteries.[18] One important question is whether the pathophysiologic mechanisms of restenosis shown in animal models also apply to the clinical situation. A number of studies show the therapeutic benefit of ET receptor antagonism by reduction of neointima formation in animal models of restenosis,[24-26] but many other pharmacologic agents that have been shown to be effective in small animals have not been reproducible in human studies, which thereby highlights the limitations of animal models.[27] In human saphenous vein used as an arterial bypass graft conduit, the roles of ET-1 and of the receptors involved with respect to the development of IH have not been studied. The purpose of this study was the investigatation of whether ET receptor antagonists can ameliorate IH in cultured human saphenous vein and, hence, the determination of whether this is effected via the ET_A receptor, ET_B receptor, or both. We investigated the following 3 compounds: bosentan, the most potent orally active mixed antagonist of ET receptors so far described;[28] BQ 123, a selective antagonist of the ET_A receptor;[29] and BQ 788, a selective antagonist of the ET_B receptor.[30] We used an organ culture of human long saphenous vein (LSV), a well-validated model of IH[31] in which, after a 14-day culture period, vessels develop a cellular neointima that has many of the characteristics of IH seen in vivo.[31]

PATIENTS AND METHODS

Culture method. For each ET receptor antagonist, segments of the LSV were obtained from a minimum of 10 patients who underwent arterial bypass grafting. The segments were transported to the laboratory in a calcium-free physiologic saline solution and prepared for culture with a method previously described.[31] Local ethical committee approval was obtained. Briefly, the excess fat and the adventitial tissue were dissected from the vessels, which were then opened longitudinally and cut into 0.5-cm lengths. The vessels were pinned, lumenal surface uppermost, with fine minuten pins (Watkins and Doncaster, Cranbrook, United Kingdom) onto a 500-μm mesh that rested on a layer of preformed Sylgard resin (Dow Corning, Seneffe, Belgium) in the the bottom of a 60 × 20–mm glass petri dish. This method of vein culture has been reported previously[31-34] and has been validated extensively in our laboratory as a representative in vitro model of the changes that occur in vein-graft IH in vivo.[31] The pinning of flat vein segments was the chosen method because of limitations on the amount of tissue that could reasonably be spared at the time of surgery. To eliminate the possibility of artifactual measurements of the vein wall, we performed an initial validation study to compare the pinning method of flat segments of vein with perfusion through the intact vein.

The mean difference of intimal thickness between the 2 methods then was evaluated. From this evaluation, we concluded that there was a highly significant level of agreement between the 2 methods of fixation.[35] Cultures were maintained in RPMI 1640 medium (Northumbria Biologicals, Cramlington, United Kingdom) and supplemented with 30% fetal calf serum (Seralab, Crawley Down, United Kingdom) for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in the air. Consecutive segments were prepared from each vessel, such that they were equivalent before randomization to the different treatment groups. In each group, 1 segment served as control, and to the others, either bosentan (10 μmol/L), BQ 123 (1 μmol/L and 3 μmol/L), or BQ 788 (1 μmol/L and 3 μmol/L) were added. Bosentan generously was provided by Roche Products Ltd (Welwyn Garden City, United Kingdom), and BQ 788 and BQ 123 were purchased from Calbiochem-Novabiochem Ltd (Nottingham, United Kingdom). All compounds were prepared in a 10% dimethyl sulfoxide solution, and the control veins received an equivalent volume of vehicle only. The culture medium and the drugs were replaced every 2 to 3 days, and, after 14 days, while still pinned in the culture dishes, the segments were fixed overnight in 10% formalin solution, processed, and embedded in paraffin. Transverse sections of 4-μm thickness were double-stained with a combined monoclonal anti–smooth muscle actin and Miller's elastin stain[36] to identify the layers of the vein wall. Mouse anti–human alpha smooth muscle actin antibody (Dako, High Wycombe, United Kingdom) was applied at a ratio of 1:400, and diaminobenzidine was used as a final reaction product. After this procedure, a Miller's elastin stain was superimposed.[37]

Measurement of neointimal thickness. The measurements of neointimal thickness were made on transverse sections of each vessel with a computerized image analysis system (Improvision, Coventry, United Kingdom). Thirty measurements were made on each vein evenly distributed across the whole section. The measurements were performed by 2 independent observers, with a high level of agreement (interobserver error). The measurements for any 1 section were found to be both consistent and reproducible (intraobserver error).[35]

Statistics. All of the summary data are expressed as median and range. The differences between the neointimal thickness of the paired control and the drug-treated veins were analyzed with a Wilcoxon signed rank test, with 95% confidence intervals.

RESULTS

Neointimal thickness. The histologic examination of the immunostained segments revealed that the control veins all developed a significant neointima composed of several layers of smooth muscle actin–positive cells after 14 days in culture (Fig 1, *A*). The vein segments that were treated with the mixed antagonist bosentan developed significantly less neointima than the paired control veins (Fig 1, *B*). The median neointimal thickness of the control veins was 16.75 μm (range, 5 to 34 μm) versus the bosentan-treated veins at 4.5 μm (range, 0 to 17 μm). The median difference was 11.5 μm, with 95% confidence intervals that were 8.75 and 15.0 (P = .001; Fig 2).

Similarly, the veins that were treated with the specific ET_B antagonist BQ 788 also showed significantly less neointimal thickening than did the control veins at both the 1-μmol/L and the 3-μmol/L concentrations. The median neointimal thickness of the control veins in this group was 28 μm (range, 22 to 61 μm) versus 16.5 μm (range, 0 to 46 μm) in the 1-μmol/L group and 14 μm (range, 5 to 49 μm) in the 3-μmol/L group. For the control group versus the 1-μmol/L BQ 788 group, the median difference was 16.0 μm, with 95% confidence intervals that were 7.5 and 28.0 (P = .008). For the control group versus the 3-μmol/L group, the median difference was 16.0 μm, with 95% confidence intervals that were 9.0 and 30.0 (P = .03; Fig 3). In addition, there was no further significant reduction in neointimal thickness in the 3-μmol/L group as compared with the 1-μmol/L group. The median difference was 3.0 μm, with 95% confidence intervals that were –12.5 and 8.5 (P = .52).

The treatment of the vein segments with the ET_A-selective antagonist BQ 123 did not have any significant effect on the reduction of neointima formation at either 1 μmol/L or 3 μmol/L. The median neointimal thickness of the control veins was 24 μm (range, 0 to 56 μm) versus 26 μm (range, 10 to 23 μm) in the 1-μmol/L group and 26 μm (range, 5 to 54 μm) in the 3-μmol/L group. The median difference was 0.0, with 95% confidence intervals that were –10.0 and 13.5, and –9.5 and 21.0, respectively. The P value was 1.00 in both comparisons (Fig 4).

DISCUSSION

Although a large number of pharmacologic agents have been shown to reduce IH in animal models, an overwhelming majority of the clinical pharmacologic studies reported to date have failed to show a significant reduction in the incidence rate of restenosis in

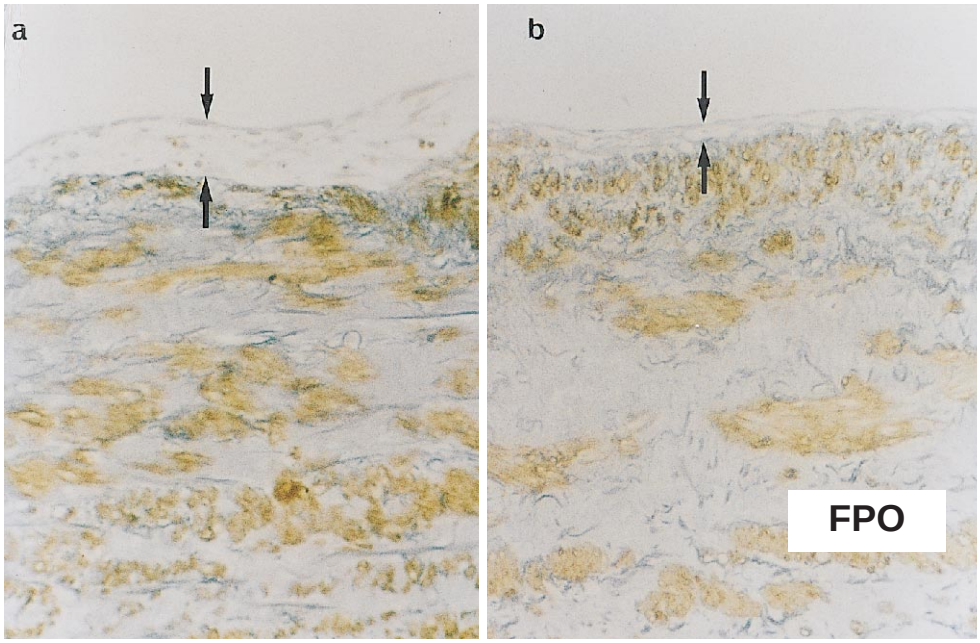

Fig 1. Representative transverse sections of long saphenous vein cultured for 14 days and stained with monoclonal anti–smooth muscle actin and Miller's elastin, which shows neointimal formation *(arrows)* at luminal surface. Control veins treated with vehicle only **(A)** developed a significantly thicker neointima than bosentan-treated veins **(B)**. (Original magnification ×160.)

humans.[38,39] The drugs that have failed in human trials include heparin, hirudin, angiotensin converting enzyme inhibitors, angiopeptin, calcium channel blockers, colchicine, and corticosteroids.[39] These results are in sharp contrast with the often promising results obtained in animal experimental models.[40] The study described here uses the precise tissue that causes the clinical problem—the human saphenous vein. The main drawback of this in vitro model is that it does not experience flow conditions, and we previously have demonstrated that vein segments cultured under conditions of laminar flow and high shear stress do not develop IH, whereas vein cultured under low or no flow conditions does develop a neointima.[41] In vivo, vein grafts have been shown to develop stenosis at sites of low or turbulent flow and hence low shear stress, where one would therefore expect to observe a locally increased production of ET-1.

A growing body of evidence shows that ET-1 plays a major role in the development of IH in human arteries and veins, and the finding that bosentan and BQ 788 significantly reduce the development of IH in organ-cultured LSV supports this view and also implies a major role for the ET_B receptor subtype in the pathogenesis of neointima formation. The percentage reduction in neointima that is achieved with bosentan (70%) and BQ 788 (50%) is considerably greater than we have found in the same model with therapeutic levels of either the angiotensin converting enzyme inhibitor losartan (30%)[42] or with low molecular weight heparin (0%).[43] The concentration of bosentan used (10 μmol/L) in this study is in the middle of the therapeutic serum levels achieved after oral administration.[44] The chosen concentrations of BQ 123 also have been shown to be associated with significant and selective ET_A receptor antagonism.[45]

The present study as it stands does not identify whether the effects of the ET receptor antagonists are on proliferation or on migration. A recent in vitro study that was performed in this laboratory showed that bosentan, BQ123, and BQ788 all significantly inhibited the proliferative response of the cultured human saphenous vein smooth muscle cells to ET-1 [unpublished observations]. Studies that investigate the effects of these drugs on migration are currently in progress.

In support of our current observations, previous studies that used balloon-injured animal arteries also have proposed a role for the ET_B receptor in neointimal formation. It has been shown that chronic administration of the nonpeptide ET_A/ET_B recep-

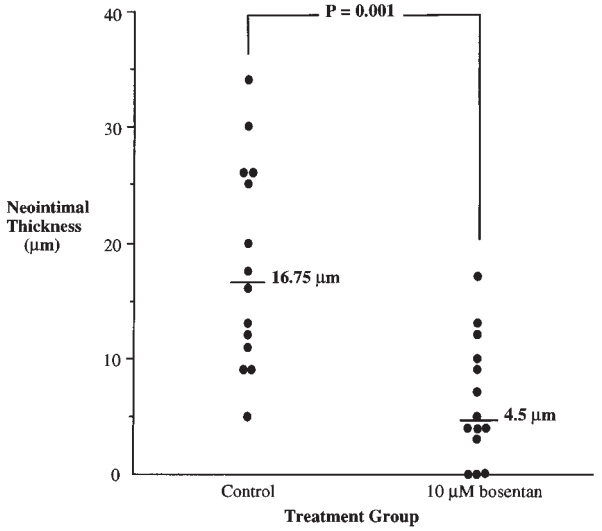

Fig 2. Scatter plot shows neointimal thicknesses of 14 paired long saphenous vein segments. Vein segments were cultured for 14 days in the absence or presence of nonselective endothelin receptor antagonist bosentan (10 μmol/L). Significance was tested with Wilcoxon signed rank test.

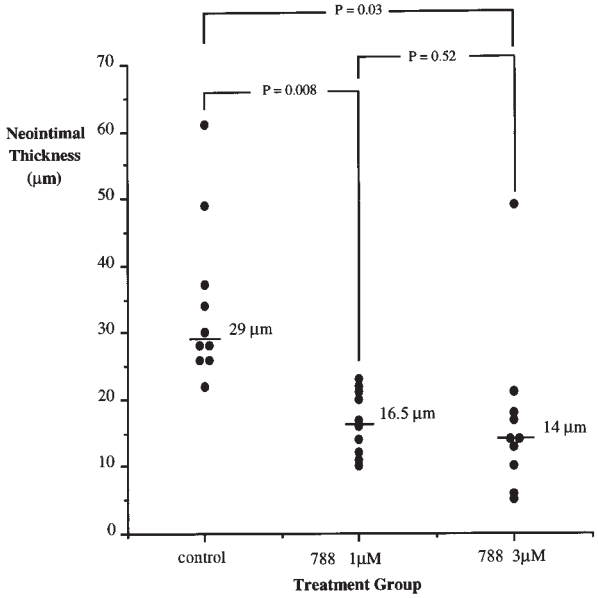

Fig 3. Scatter plot shows neointimal thicknesses of 10 paired long saphenous vein segments cultured in the absence or presence of endothelin-B selective antagonist BQ 788 (3 μmol/L and 1 μmol/L). Significance was tested with Wilcoxon signed rank test.

tor antagonist SB 209670 ameliorates the neointima formation observed in the rat carotid artery.[18] Furthermore, in agreement with the present study, the selective ET_A receptor antagonist BQ 123 was found to be ineffective in this role, an outcome that implicates the ET_B receptor subtype in the genesis of neointima formation.[26] Another study with balloon-injured rabbit carotid arteries[22] showed a markedly elevated ET-1 immunoreactivity in injured arteries, together with a doubling of ET_B receptor density that was expressed predominantly in the neointima. The same study also showed that the chronic intravenous administration of BQ 123 at concentrations that were sufficient to antagonize the ET_A receptors had no effect on neointima formation. These observations also highlight the increased relative importance of the ET_B receptor subtype in the synthetic phenotype of smooth muscle cells that comprise the fibroproliferative stages of IH and restenosis. The above results and those of the present study suggest that ET inhibition may have an important part to play in the amelioration of IH in human saphenous vein. The results also raise the possibility that selective ET-1 antagonists could serve as novel therapeutic agents in the control of restenosis.[27] Many drug companies now are developing orally active ET antagonists with the intention of producing drugs with a therapeutic potential in man. The preventing

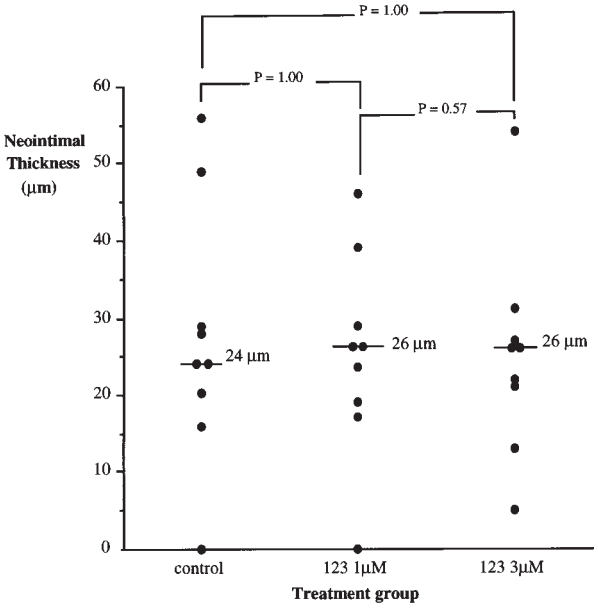

Fig 4. Scatter plot shows neointimal thicknesses of 10 paired long saphenous vein segments cultured in the absence or presence of endothelin-A selective antagonist BQ 123 (3 μmol/L and 1 μmol/L). Significance was tested with Wilcoxon signed rank test.

of the possible pathophysiologic effects of ET at its receptors with selective antagonists might represent a major advance in pharmacotherapy. Some of the agents are receptor specific for the ET_A or the ET_B subtype, but others are nonspecific. We are currently investigating the time course of the expression of ET_A and ET_B in sequentially cultured vein segments between day 0 and day 14 with PCR, radioligand binding, and in-situ hybridization.

The present study examined only 1 time point—14 days. We previously have shown that neointimal thickening reaches a maximum between day 10 and day 14 in culture.[31] Recently, we have shown that ET-1 production with cultured control veins peaks around day 6, with a rapid "fall off" by day 12 [unpublished results]. Taken together, these observations suggest that a continuous therapy during the 14 days would probably be necessary to achieve a durable response.

It has been shown that infusion of ET-1 into the brachial artery of healthy men causes a venoconstriction that can be abolished by the coinfusion of the ET_A selective antagonist BQ 123.[46] The same study also showed that the infusion of BQ 123 alone caused a progressive dilatation with a resultant increase in the forearm blood flow rate of 64%. These observations suggest that the endogenous generation of ET-1 contributes to the maintenance of vascular tone in states of normal and elevated blood pressure. Chronically elevated ET-1 levels and the activation of the ET_A receptor also have been shown to play a direct and contributary role in the progression of chronic heart failure in rabbits.[47] Therefore, specific ET_A antagonists may have potential as vasodilators in the treatment of diseases that are associated with vasoconstriction, such as hypertension and chronic heart failure.

In contrast, the observations that were made in this study suggest a direct role for the ET_B receptor in the development of IH. Therefore, the specific antagonism of the ET_B receptor may attenuate the development of human saphenous vein graft stenosis, without the undesirable effects of ET_A antagonism, which might result from the administration of a nonselective ET receptor antagonist.

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